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ORIGINAL PAPER

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# (*S*)-1-Phenylethanol dehydrogenase of *Azoarcus* sp. strain EbN1, an enzyme of anaerobic ethylbenzene catabolism

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**Abstract** The initial steps in the anaerobic oxidation of the aromatic hydrocarbon ethylbenzene by denitrifying bacteria are two sequential dehydrogenation reactions of ethylbenzene to (*S*)-1-phenylethanol and further to acetophenone. The enzyme catalysing the second oxidation step, (*S*)-1-phenylethanol dehydrogenase, was analysed in the denitrifying bacterium *Azoarcus* sp. strain EbN1. An $NAD^+$-dependent 1-phenylethanol dehydrogenase for each of the enantiomers of 1-phenylethanol was identified in this bacterium; the two enzymes were induced under different growth conditions. (*S*)-1-phenylethanol dehydrogenase from ethylbenzene-grown cells was purified and biochemically characterised. The enzyme is a typical secondary alcohol dehydrogenase and consists of two subunits of 25.5 kDa. The enantioselective enzyme catalyses the oxidation of (*S*)-1-phenylethanol or the reduction of acetophenone and is inhibited by high concentrations of (*R*)-1-phenylethanol. The enzyme exhibits low apparent $K_m$ values for (*S*)-1-phenylethanol and acetophenone and is rather substrate-specific, using only a few chemically similar secondary alcohols, such as 1-phenylpropanol and isopropanol.

**Keywords** Anaerobic catabolism · Ethylbenzene · 1-Phenylethanol · Acetophenone · Short-chain alcohol dehydrogenase

## Introduction

Three denitrifying *Azoarcus* sp., strains EbN1, PbN1 and EB-1, are known to degrade the aromatic hydrocarbon ethylbenzene as sole carbon source and electron donor in the absence of oxygen (Rabus and Widdel 1995; Ball et al. 1996). Whereas strain EB-1 utilises ethylbenzene as the only aromatic hydrocarbon (Ball et al. 1996), strain PbN1 grows with either ethylbenzene or *n*-propylbenzene, and strain EbN1 with either ethylbenzene or toluene (Rabus and Widdel 1995). The degradation pathway of toluene in strain EbN1 is identical to that used by other anaerobic toluene-degrading bacteria and involves the formation of benzylsuccinate from toluene and fumarate as the first step (Rabus and Heider 1998; Heider et al. 1999). However, anaerobic degradation of ethylbenzene (and *n*-propylbenzene) proceeds via a completely different pathway in all known denitrifying strains (reviewed in Heider et al. 1999). Ethylbenzene catabolism is initiated by an oxygen-independent oxidation of the methylene group to yield 1-phenylethanol, which is further oxidised to acetophenone (Rabus and Widdel 1995; Ball et al. 1996; Rabus and Heider 1998; Champion et al. 1999; Johnson and Spormann 1999). Further degradation of acetophenone apparently involves carboxylation to benzoylacetate, activation to the thioester and thiolytic cleavage, yielding acetyl-CoA and benzoyl-CoA as intermediates (Fig. 1; Ball et al. 1996; Champion et al. 1999). The first two steps of anaerobic ethylbenzene degradation in *Azoarcus* sp. strain EB-1 were recently investigated in more detail (Johnson and Spormann 1999). Whereas the enzymes involved have not been purified and characterised, stereospecific formation of (*S*)-1-phenylethanol as intermediate was reported, and the presence of an (*S*)-1-phenylethanol dehydrogenase was detected in extracts of ethylbenzene-grown cells of strain EB-1 (Johnson and Spormann 1999).

A number of (*S*)- and (*R*)-1-phenylethanol dehydrogenases have been characterised to date from different microbial sources, together with some other enzymes of biotechnological interest for specific production of the (*S*)- or (*R*)-enantiomers of 1-phenylethanol. For example, $NADP^+$-dependent (*R*)-1-phenylethanol-dehydrogenases were purified from *Lactobacillus* species (Hummel 1990, 1997, 1999). These enzymes belong to the short-chain

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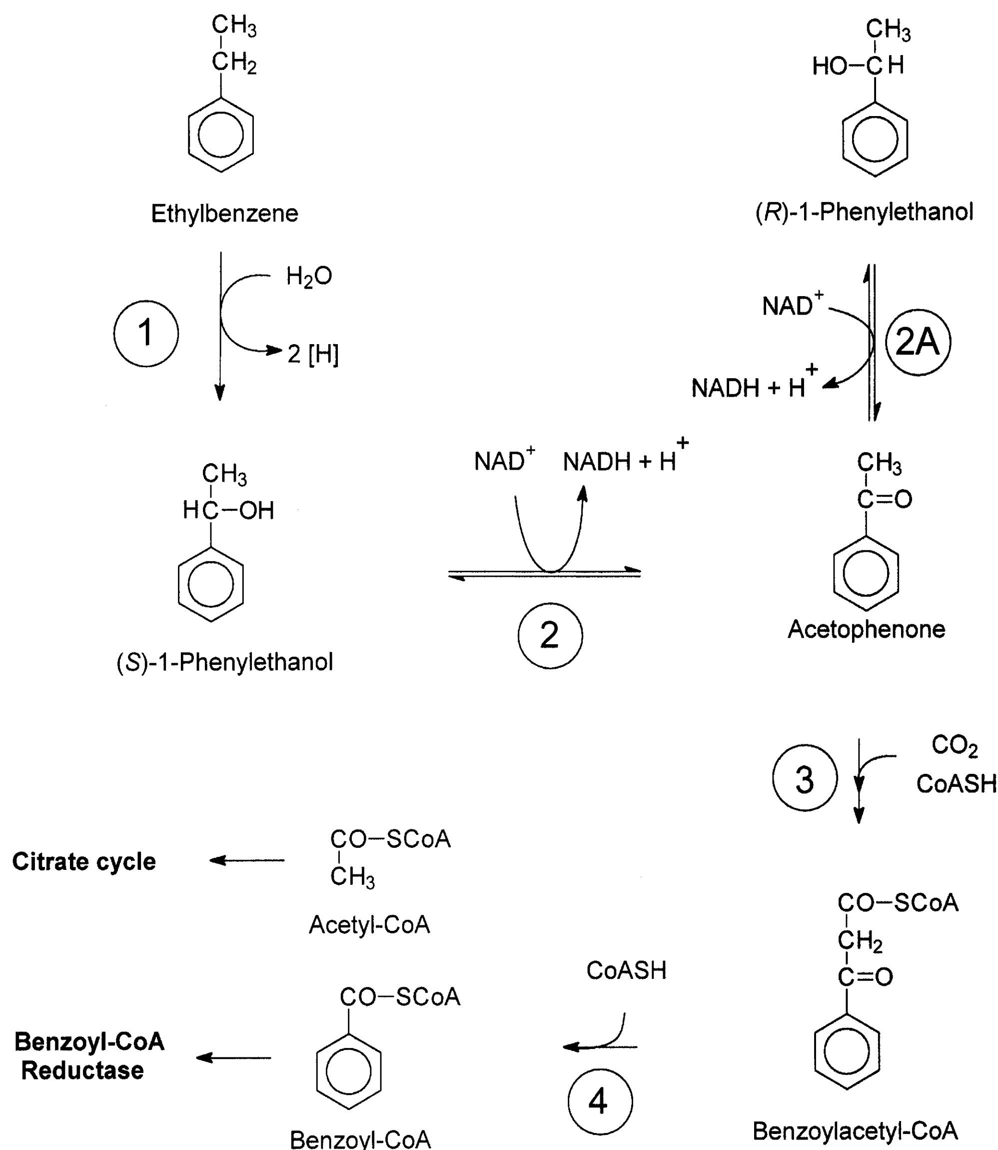


**Fig. 1** Proposed catabolic pathway of ethylbenzene. Enzymes: *1* ethylbenzene dehydrogenase, *2* (*S*)-1-phenylethanol dehydrogenase, *2A* (*R*)-1-phenylethanol dehydrogenase, *3* acetophenone carboxylase and benzoylacetate-activating enzyme, *4* benzoylacetyl-CoA thiolase

family of alcohol dehydrogenases, whereas another purified $NAD^+$-dependent (*S*)-1-phenylethanol-dehydrogenase from *Rhodococcus erythropolis* belongs to the medium-chain, $Zn^{2+}$-containing alcohol dehydrogenases (Hummel 1997). $NAD^+$-dependent (*S*)- and (*R*)-1-phenylethanol dehydrogenase activities were also found in crude extracts of an *Arthrobacter* sp. grown aerobically on racemic 1-phenylethanol, but the enzyme(s) were not purified and characterised further (Cripps et al. 1978). Stereospecific reduction of acetophenone to (*S*)-1-phenylethanol is also catalysed as a side reaction by a phenylacetaldehyde reductase from a *Corynebacterium* sp. (Itoh 1997) and by a “carbonyl reductase” from *Candida parapsilosis* (Peters 1993). Finally, a naphthalene dioxygenase from a *Pseudomonas* sp. is known to catalyse the non-specific oxidation of ethylbenzene via (*S*)-1-phenylethanol and acetophenone to 2-hydroxyacetophenone (Lee and Gibson 1996).

In this communication, we report the presence of 1-phenylethanol dehydrogenases in cells of *Azoarcus* sp. strain EbN1 grown anaerobically on ethylbenzene and different intermediates of the postulated metabolic pathway. An (*S*)-1-phenylethanol dehydrogenase apparently catalysing the second step of anaerobic ethylbenzene metabolism was purified and further characterised. The enzyme should also provide a source of auxiliary enzyme to reconstitute the initial ethylbenzene catabolic pathway in vitro.

## Materials and methods

### Cell growth and preparation of extract

Denitrifying *Azoarcus* sp. strain EbN1 was isolated by Rabus and Widdel (1995) from a freshwater enrichment culture on ethylbenzene. Bacteria were grown in 1- to 2-l cultures as described previously (Rabus and Widdel 1995; Rabus and Heider 1998). These

were harvested anoxically during the exponential growth phase at OD values of 0.30–0.34. Cultures on ethylbenzene were also grown in fed-batch mode in a 200-l fermenter, which allowed exponential growth up to optical densities of 2.0. Nitrate was fed continuously from a 1.5 M $NaNO_3$ stock solution with an exponentially increasing and growth-limiting rate, and ethylbenzene portions of 0.4 mol were discontinuously supplemented when the optical density of the culture increased by 0.4. Some experiments were also performed with cells harvested in the stationary phase, either from small-scale experiments (OD values of 0.41–0.45) or from a fermenter run in which feeding was stopped at an OD of 1.5. For extract preparation, 10 g (wet mass) of cells were suspended in 20 ml of basal buffer [25 mM Tris/HCl, pH 8, 2 mM $MgCl_2$, 0.2 mM dithioerythritol, 10% (w/v) glycerol]. Cells were lysed by two passages through a French pressure cell at 137 MPa. Cell debris and membranes were removed by ultracentrifugation (1 h 100,000×*g*). Enzyme activities in aerobically and anaerobically prepared extracts reported in this communication were identical and enzyme activity was not affected when dithioerythritol or $MgCl_2$ were omitted from the buffer.

### Enzyme assays

Activity of (*R*)- and (*S*)-1-phenylethanol dehydrogenase was measured at 30 °C in 100 mM Tris-Cl buffer (pH 8) containing 2 mM $MgCl_2$ and 0.5 mM $NAD^+$. Extract or column fractions (10–50 µl per ml assay) were added, and the reaction was started by addition of 1 mM substrate. For assaying the reverse reaction, $NAD^+$ was replaced by NADH and the reaction was started with 1 mM acetophenone. To determine reaction stoichiometries, (*S*)-1-phenylethanol or acetophenone was added in low concentrations (40 µM each) to the assays and allowed to react to completion. Stoichiometries were calculated from the applied substrate concentrations and the corresponding changes in pyridine nucleotide concentrations. To assess the pH optimum of purified (*S*)-1-phenylethanol dehydrogenase, the enzyme test was performed in the following buffers (100 mM each): citric acid/$Na_2HPO_4$ (pH 3.5–6.0), Na-phosphate buffer (pH 6.0–8.0), and boric acid/NaOH buffer containing 100 mM KCl (pH 8.0–9.0). For determination of the reaction kinetics, the concentration of one substrate was varied, while the other was provided at a constant concentration ($\geq 10 \times K_m$). The range of substrate concentrations used was 1–30 µM for 1-phenylethanol and acetophenone, 10–500 µM for $NAD^+$ and 0.6–10 µM for NADH.

### Purification of (*S*)-1-phenylethanol dehydrogenase

All purification steps were carried out under oxic conditions at 6 °C. Extract of cells of strain EbN1 grown on ethylbenzene (12 ml of a 100,000×*g* supernatant) was applied to a DEAE-Sepharose column (Pharmacia; diameter, 2.2 cm; volume, 30 ml), which had been equilibrated with basal buffer. The column was washed with the same buffer at a flow rate of 1 ml $min^{-1}$ for four column volumes. The column was eluted with a step gradient to 30 mM KCl, and a subsequent linear gradient from 30 to 200 mM KCl in basal buffer over 170 ml. Fractions of 5 ml were collected, and the activities of (*S*)- and (*R*)-1-phenylethanol dehydrogenase were monitored. (*S*)-1-phenylethanol dehydrogenase activity eluted between 40 and 90 mM KCl, whereas (*R*)-1-phenylethanol dehydrogenase activity eluted between 65 and 95 mM KCl. The fractions eluting between 40 and 60 mM KCl contained only (*S*)-1-phenylethanol dehydrogenase activity and were combined. The obtained pool of 50 ml contained 36% of the total (*S*)-1-phenylethanol dehydrogenase activity and was used for further purification. Ammonium sulfate was added to a final concentration of 1 M, and the solution was applied to a TSK-butyl-fractogel column (Merck; 1.6 cm diameter; 15 ml volume) equilibrated with basal buffer containing 1 M ammonium sulfate. The column was washed with 70 ml of the same buffer and eluted with a step gradient to 0.5 M ammonium sulfate and a subsequent linear gradient from 0.5 to 0 M ammonium sulfate in basal buffer over six column volumes. Fractions of 3 ml were collected. (*S*)-1-phenylethanol dehydrogenase activity eluted from the column after the end of the gradient during washing with basal buffer in a volume of 12 ml, corresponding to 19% of the initial activity. The active fractions were pooled and concentrated by ultrafiltration with an exclusion limit of 30 kDa (Amicon 8400, with a PM-30 membrane). No loss of activity was recorded during the concentration step. Small aliquots (200 µl) of the concentrated major pool of (*S*)-1-phenylethanol dehydrogenase activity were further purified by gel filtration on a Superose 6 column (Pharmacia; 1 cm diameter; 20 ml volume). The column, which was simultaneously used to determine the molecular mass of the native enzyme, was calibrated with ferritin (440 kDa), catalase (232 kDa), alcohol-dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa ) as molecular mass markers. Exclusion and inclusion volumes were determined with blue dextran and vitamin $B_{12}$, respectively. The column was equilibrated and eluted with basal buffer containing 100 mM NaCl at a flow rate of 0.2 ml $min^{-1}$, and fractions of 0.4 ml were collected. (*S*)-1-phenylethanol dehydrogenase eluted after 16.25 ml in a volume of 1.6 ml. Active fractions from this step were pooled, concentrated and stored at –70 °C.

### Other methods

Protein concentrations were determined by the Coomassie-dye binding method with bovine serum albumin as standard, and discontinuous SDS-PAGE with 11% (w/v) polyacrylamide gels was carried out according to standard procedures (Coligan et al. 1999). Molecular mass standards were phosphorylase b, bovine serum albumin, ovalbumin, lactate dehydrogenase and carbonic anhydrase. Purified enzyme (60 µg) was separated by two-dimensional gel electrophoresis, using the Immobiline Dry Strip system (linear pH gradient 3–10, 11 cm; Pharmacia) for the first dimension according to the manufacturer's protocol. The focused proteins in the gel strips were then separated in the second dimension by discontinuous SDS-PAGE as described above. For protein microsequencing, purified enzyme was blotted on a PVDF membrane (ProBlott; Applied Biosystems) after gel electrophoresis using a semi-dry blotting device (Semi-Phor TE77; Amersham Pharmacia Biotech) as described by Matsudaira (1982). Amino acid sequences were obtained via Edman-degradation (Procise 492 Sequencer; Applied Biosystems). Chemicals were purchased from Sigma, Fluka or Merck and were of the highest available purity.

## Results

### 1-Phenylethanol dehydrogenase isoenzymes in cells of strain EbN1

Cell extracts of strain EbN1 grown on ethylbenzene and harvested during the exponential growth phase were tested for the presence of $NAD^+$- or $NADP^+$-coupled 1-phenylethanol dehydrogenases. Activity was only observed in assays with $NAD^+$ as electron acceptor, not with $NADP^+$. Enzyme activity was observed with both enantiomers of 1-phenylethanol, and no reaction occurred with the primary alcohol 2-phenylethanol (data not shown). A specific activity of (*S*)-1-phenylethanol dehydrogenase of 67 nmol $min^{-1}$ (mg protein)$^{-1}$ was recorded, whereas the same extracts oxidised (*R*)-1-phenylethanol at a 12-fold lower rate (Table 1). Ethylbenzene-grown cells harvested in the stationary phase contained similar specific activities for (*S*)-1-phenylethanol, but twofold increased activities of (*R*)-1-phenylethanol dehydrogenase, compared to cells harvested in the exponential growth phase (Table 1).

**Table 1** 1-Phenylethanol dehydrogenase activities in cells of strain EbN1 grown anaerobically with different organic substrates. Mean activities of at least two measurements are shown; the obtained standard deviations were <10%

| Growth substrate | Specific activity [nmol min$^{-1}$ (mg protein)$^{-1}$] | |
|---|---|---|
| | (*R*)-1-phenylethanol dehydrogenase | (*S*)-1-phenylethanol dehydrogenase |
| Ethylbenzene (exponential) | 6 | 67 |
| Ethylbenzene (stationary) | 11 | 73 |
| (*S*)-1-phenylethanol | 125 | 63 |
| (*R*)-1-phenylethanol | 90 | 46 |
| (*S/R*)-1-phenylethanol | 104 | 27 |
| Acetophenone | 88 | 42 |
| Benzoate | 3 | <0.1 |
| Acetate | 4 | <0.1 |

Furthermore, enzyme activities were examined in extracts of cells grown on assumed intermediates of the ethylbenzene metabolism in strain EbN1. Cells grown on (*R*)- or (*S*)-1-phenylethanol or acetophenone contained (*S*)-1-phenylethanol dehydrogenase of similar specific activity as ethylbenzene-grown cells: the values were almost identical in (*S*)-1-phenylethanol-grown cells and were only slightly decreased in the other cell batches tested. However, the specific activity of (*R*)-1-phenylethanol dehydrogenase was drastically increased in all these cell extracts, even those grown on the (*S*)-enantiomer (Table 1). The presence of a highly efficient inducible isoenzyme for (*R*)-1-phenylethanol oxidation is consistent with a relatively short doubling time of 8 h for growth on (*R*)-1-phenylethanol, compared to 11 h for growth on ethylbenzene, and 13 h on (*S*)-1-phenylethanol. Almost no activity of either 1-phenylethanol dehydrogenase isoenzyme was present in extracts of benzoate- or acetate-grown cells (Table 1).

## Purification of (*S*)-1-phenylethanol dehydrogenase

Proteins in extracts of ethylbenzene-grown cells were separated by chromatography on DEAE-sepharose, and the activities of (*S*)- and (*R*)-1-phenylethanol dehydrogenases were monitored in the eluate fractions. Enzymes oxidising the different 1-phenylethanol enantiomers were separated by this chromatographic step. Most of the (*S*)-1-phenylethanol dehydrogenase activity eluted between 40 and 60 mM KCl with 36% yield, and a mixture of (*R*)- and (*S*)-1-phenylethanol dehydrogenase eluted between 65 and 95 mM KCl with 130% (apparent) yield of the (*R*)-specific isoenzyme and 21% yield of the (*S*)-specific isoenzyme. The small amount of (*R*)-1-phenylethanol dehydrogenase was not purified further. Purification of (*S*)-1-phenylethanol dehydrogenase was continued with the fractions containing the enzyme without contamination by (*R*)-1-phenylethanol dehydrogenase. These were pooled, mixed with ammonium sulfate to a concentration of 1 M and separated by chromatography on a TSK-butyl-fractogel column. (*S*)-1-phenylethanol dehydrogenase eluted between 20 and 0 mM ammonium sulfate with a yield of 19%. The active fractions were pooled and concentrated by ultrafiltration; they appeared >90% pure after SDS-PAGE (Fig. 2). An aliquot was applied on a Superose-6 gel filtration column. Active fractions from this step were pooled, concentrated and stored at –70 °C. The gel filtration step caused some loss of activity and reduced the yield considerably, but removed the last minor contaminants (Fig. 2). The enzyme was 83-fold enriched and essentially pure after these three chromatographic steps. A summary of the purification procedure is given in Table 2.

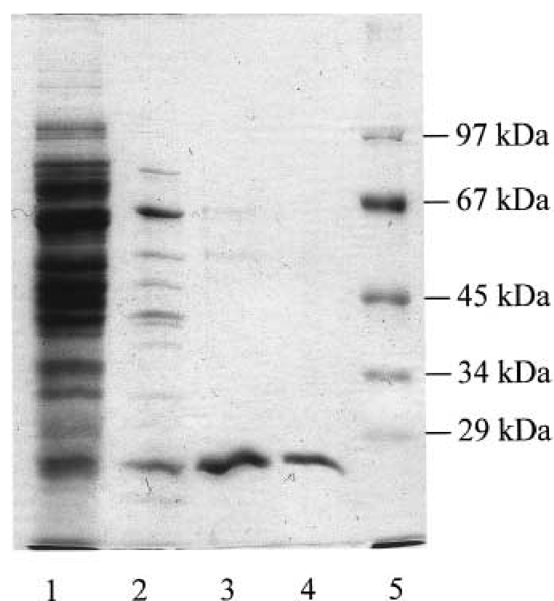


**Fig. 2** SDS-PAGE of protein fractions containing (*S*)-1-phenylethanol dehydrogenase activity obtained during purification of the enzyme. An 11% (w/v) polyacrylamide gel is shown that was stained with Coomassie blue. Lanes: *1* cell extract, *2* pooled fractions from DEAE sepharose chromatography, *3* pooled fractions from butyl-TSK chromatography, *4* pooled fractions from gel filtration, *5* molecular mass markers. The masses of marker proteins are indicated in the *right margin*

## Molecular and catalytic properties of (*S*)-1-phenylethanol dehydrogenase

(*S*)-1-phenylethanol dehydrogenase from ethylbenzene-grown cells consisted of a single subunit of 25.5±0.5 kDa

**Table 2** Purification of (*S*)-1-phenylethanol dehydrogenase. Note that actual yield values and corrected yield values (in *parentheses*) are given. The corrected values include the amounts of enzyme in side fractions that were not used for subsequent purification steps

| Purification step | Volume (ml) | Total activity (µmol $min^{-1}$) | Total protein [mg] | Specific activity (µmol $min^{-1}$ $mg^{-1}$) | Yield (%) | Enrichment (-fold) |
|---|---|---|---|---|---|---|
| Extract | 12 | 29 | 623 | 0.046 | 100 | 1 |
| DEAE-sepharose | 50 | 10.5 | 23 | 0.46 | 36 (57) | 10 |
| Butyl-fractogel | 12 | 5.5 | 1.8 | 3.1 | 19 (35) | 67 |
| Gel filtration | 1.6 | 0.3 | 0.07 | 3.8 | 1 (11) | 83 |

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EbN1 1-PEDH:   1 TQRLKDKLAVITGGANGIGRAIAERF 26
Zm 3-OA-ACPR:  1  MRFQDKIVVITGGGNGMGKAAAERF 25 (AAG02168)
Bm GDH:        2 YTDLKDKVVVITGGSTGLGRAMAVRF 27 (P39484)
At ADH-like:  33 SRKLEGKVAVITGGASGIGKATAEEF 58 (BAB01222)
Ta GDH-like:   2 FSDLRDKVVIVTGASMGIGRAIAERF 27 (CAC11888)
Pp DHD-DH:     3 NKRFQDKTAVITGAAQGIGRRVAERM 26 (AAD31450)
Rsp DHD-DH:    1  MRLKDEVVLVTGGCAGLGRAIVDRF 25 (AAB07754)
Bt ADH:        1  MSIEQKTAIVTGGANGIGKAIARAF 25 (BAA94092)
Ta 3-OA-ACPR:  2 SGKLEGKIALITGASKGLGRAIAEKF 27 (CAC11396)
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**Fig. 3** N-terminal amino acid sequence of (*S*)-1-phenylethanol dehydrogenase. The N-terminal sequence of (*S*)-1-phenylethanol dehydrogenase is given in the *first line*, those of the most similar proteins found in the data base are shown for comparison. Identical and chemically similar amino acids are printed in *bold*. *Zm*, *Zymomonas mobilis*; *Bm*, *Bacillus megaterium*; *At*, *Arabidopsis thaliana*; *Ta*, *Thermoplasma acidophilum*; *Pp*, *Pseudomonas putida*; *Rsp*, *Rhodococcus* species; *Bt*, *Bacillus thermoleovorans*; *3-OA-ACPR*, 3-oxoacyl-ACP reductase; *GDH*, glucose 1-dehydrogenase; *ADH*, short-chain alcohol dehydrogenase; *DHD-DH*, *cis*-dihydrodiol-dehydrogenase. *Numbers* indicate the positions of the first and last amino acids in the proteins. Database accession numbers are given in *parentheses*

and exhibited a *p*I of 6.6, as revealed by SDS-PAGE and two-dimensional gel electrophoresis (data not shown). The N-terminal sequence of the subunit was determined and compared with the protein sequence database. It was most similar to several short-chain alcohol dehydrogenases, which typically catalyse the oxidation of secondary alcohols (Fig. 3). The apparent native molecular mass of the enzyme was 50±5 kDa, as determined by gel filtration. From the values obtained with purified enzyme, an $\alpha_2$ (homodimer) composition can be assumed, as typically found for short-chain alcohol dehydrogenases. UV-visible spectroscopic analysis of the purified enzyme showed an absorption maximum at 280 nm with a shoulder at 290 nm, possibly originating from tryptophan residues, but no further absorption characteristics at longer wavelengths. The determined absorption coefficient at 280 nm ($\varepsilon_{280}$) was 94.8 $mM^{-1}$ $cm^{-1}$ (relative to homodimeric enzyme).

The purified enzyme catalysed the $NAD^+$-dependent oxidation of (*S*)-1-phenylethanol as well as the NADH-dependent reduction of acetophenone. The apparent pH optimum of the forward reaction was at pH 8.0, whereas the optimum for acetophenone reduction activity was at pH 4.0. Substrate-limited assays of the forward and reverse reactions yielded an (*S*)-1-phenylethanol:$NAD^+$ stoichiometry of 1.1 and an acetophenone:NADH stoichiometry of 0.7. Linear Lineweaver-Burk plots were obtained with up to 500 µM $NAD^+$, 10 µM NADH, 30 µM (*S*)-1-phenylethanol, and 8 µM acetophenone. The calculated $k_{cat}$ values per holoenzyme ($\alpha_2$) were 2.0 $s^{-1}$ for alcohol oxidation, and 1.6 $s^{-1}$ for ketone reduction (both measured at pH 8). Apparent $K_m$ values of the forward direction were 1.6±0.3 µM for (*S*)-1-phenylethanol (at 0.5 mM $NAD^+$) and 51±6 µM for $NAD^+$ (at 1 mM (*S*)-1-phenylethanol). Apparent $K_m$ values of the reverse direction were 1.0±0.04 µM for acetophenone (at 0.25 mM NADH), and 3.3±0.5 µM for NADH (at 10 µM acetophenone). Acetophenone inhibited its own reduction at higher concentrations, starting at 8 µM. No activity was measurable with (*R*)-1-phenylethanol as substrate. Racemic (*R*,*S*)-1-phenylethanol was only oxidised at 36% of the rate of (*S*)-1-phenylethanol, and (*S*)-1-phenylethanol oxidation was completely inhibited in the presence of tenfold higher concentrations of the (*R*)-enantiomer (Table 3). Substrate specificity of purified (*S*)-1-phenylethanol dehydrogenase was tested in enzyme assays with a number of substrate analogues. No activity was recorded with primary alcohols, such as ethanol, 1-propanol, 1-butanol, 2-phenylethanol or benzyl alcohol, whereas the enzyme showed a similar rate with (*R*,*S*)-1-phenylpropanol as with racemic

**Table 3** Substrate specificity and inhibition of (*S*)-1-phenylethanol dehydrogenase. The reference value of specific activity (100%) was 4.0 µmol $min^{-1}$ (mg protein)$^{-1}$. Only values obtained with tested secondary alcohols are shown. Primary alcohols were not oxidised by the enzyme

| Substrate | Relative activity (%) |
|---|---|
| (*S*)-1-Phenylethanol | 100 |
| Mixtures of (*S*)- and (*R*)-1-phenylethanol | |
| (molar ratio 1:1) | 36 |
| (molar ratio 1:10) | <1 |
| (*S*,*R*)-1-Phenylpropanol | 53 |
| Isopropanol | 3 |
| Isoamyl alcohol | 0 |

1-phenylethanol and had a low but definite activity with isopropanol (Table 3). The influence of metal ion chelators and thiol reagents on (*S*)-1-phenylethanol-dehydrogenase was studied in enzyme assays without added $MgCl_2$. The presence of 10 mM EDTA (10 mM), 1 mM pyrazole or 1 mM 1,10-phenanthroline in enzyme assays did not affect the activity, indicating that the enzyme does not contain easily accessible bivalent metal ions. Likewise, (*S*)-1-phenylethanol oxidation was not inhibited by 0.1 mM 5, 5′-dithiobis-(2-nitrobenzoic acid), 1 mM 4-hydroxymercuribenzoic acid or 1 mM N-ethylmaleimide, indicating that no accessible cysteine residues are required for catalysis.

## Discussion

In this study, we analysed the induction pattern and substrate specificity of the second enzyme of the proposed pathway of anaerobic ethylbenzene metabolism, (*S*)-1-phenylethanol dehydrogenase. $NAD^+$-dependent isoenzymes catalysing the oxidation of (*S*)- or (*R*)-1-phenylethanol to acetophenone were identified in cells of strain EbN1 grown on different organic substrates. The recorded activities in cell extracts are sufficient to explain the observed growth rates on the respective substrates. The doubling time of 11 h on ethylbenzene and a growth yield of 79 g dry cell mass per mol ethylbenzene dissimilated (Rabus 1995) require a minimum activity of 27 nmol $min^{-1}$ (mg protein)$^{-1}$ (assuming a protein content of 50% in dry mass). The stereo-specificity of the prevalent isoenzymes in ethylbenzene-grown cells is consistent with a catabolic pathway via (*S*)-, but not via (*R*)-1-phenylethanol. The (*S*)-enantiomer of 1-phenylethanol was also previously reported as an intermediate of anaerobic ethylbenzene metabolism in another *Azoarcus* sp., strain EB-1 (Johnson and Spormann 1999).

Whereas only (*S*)-1-phenylethanol dehydrogenase activity was detected in ethylbenzene-degrading *Azoarcus* sp. strain EB-1 (Johnson and Spormann 1999), cells of strain EbN1 apparently synthesise differentially regulated (*R*)- and (*S*)-1-phenylethanol dehydrogenases during growth on other substrates. Activity of (*R*)-1-phenylethanol dehydrogenase is very low in ethylbenzene-grown cells and apparently increases about two-fold when cells become stationary, but this isoenzyme is present in much higher amounts in cells grown on either of the 1-phenylethanol enantiomers or on acetophenone. Remarkably, the highest (*R*)-1-phenylethanol dehydrogenase activities were detected in (*S*)-1-phenylethanol-grown cells, which indicates that the substrate, (*R*)-1-phenylethanol, is probably not the true inducer of synthesis of this isoenzyme. Since acetophenone is apparently more toxic for the organism (growth inhibition at >2.6 mM) than (*R*)- and (*S*)-1-phenylethanol (growth inhibition at >8 mM; Zengler 1996), the high 1-phenylethanol dehydrogenase activities for the "wrong" enantiomer may be explained by a possible additional function in detoxifying surplus acetophenone by reduction to 1-phenylethanol.

(*S*)-1-phenylethanol dehydrogenase of *Azoarcus* sp. strain EbN1 is a soluble protein and catalyses the oxidation of (*S*)-1-phenylethanol with $NAD^+$ as electron acceptor and the reduction of acetophenone with NADH as electron donor. Low apparent $K_m$ values for (*S*)-1-phenylethanol and acetophenone indicate that conversion of these compounds is indeed the physiological function of the enzyme. The enzyme also oxidises some secondary alcohols of similar chemical structure as 1-phenylethanol, whereas no primary alcohols are oxidised. (*R*)-1-phenylethanol is not metabolised by the enzyme, but inhibits (*S*)-1-phenylethanol oxidation in high concentrations. (*S*)-1-phenylethanol dehydrogenase belongs to the family of short-chain alcohol dehydrogenases, judging by its subunit size, composition and N-terminal sequence. Some catalytic properties of (*S*)-1-phenylethanol dehydrogenase are also typical for this enzyme family, such as the apparent absence of metal ions in the active enzyme, lack of essential SH-groups and specificity for secondary alcohols. Compared to other purified 1-phenylethanol dehydrogenases (Hummel 1999), the characterised enzyme is the first $NAD^+$-dependent short-chain alcohol dehydrogenase specific for (*S*)-1-phenylethanol.

Proteins specifically induced during growth of strain EbN1 on acetophenone were recently detected by two-dimensional gel electrophoresis (Champion et al. 1999), and N-terminal amino acid sequencing revealed a short-chain alcohol dehydrogenase among these proteins. However, the N-terminal sequence, isoelectric point and molecular mass of this protein were different from that of the presently purified enzyme. Therefore, the previously reported acetophenone-induced protein may well represent the recorded (*R*)-1-phenylethanol dehydrogenase or another isoenzyme of (*S*)-1-phenylethanol dehydrogenase.

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